Purification and properties of a phenol sulphotransferase from *Euglena* using L-tyrosine as substrate

Tekchand SAIDHA and Jerome A. SCHIFF*

Photobiology Group, Biology Department, Brandeis University, Waltham, MA 02254, U.S.A.

A purification procedure based on $(\text{NH}_4)_2\text{SO}_4$ precipitation, and chromatography on Affi-Gel Blue, DEAE-cellulose, hydroxyapatite and Bio-Gel P-60 yields a stable 6400-fold-purified active monomeric phenol (tyrosine) sulphotransferase of 26 kDa from W_{10}BSmL , an aplastidic mutant of *Euglena gracilis* var. *bacillaris*. The apparent K_m for adenosine 3'-phosphate 5'-phosphosulphate (PAPS) is 15 μM (60 μM tyrosine as substrate); adenosine 5'-phosphosulphate is inactive. L-Tyrosine gave the lowest apparent K_m (33 μM) (with PAPS at 30 μM), but tyrosine esters, tyrosinamide, L-*p*-hydroxyphenylglycine and a number of tyrosine dipeptides were also active, with higher K_m values. Nitrophenols (*m*- and *p*-) and chlorophenols (*o*-, *m*- and *p*-) were active, with

higher K_m values than for tyrosine. D-Tyrosine was inactive as a substrate, as was D-*p*-hydroxyphenylglycine and a number of other tyrosine derivatives lacking the carboxy carbonyl or the amino group, or having extra ring substituents or the hydroxy group in the wrong position. Adenosine 3',5'-bisphosphate and tyrosine *O*⁴-sulphate, products of the enzyme reaction with PAPS and tyrosine as substrates, showed competitive ($K_i = 20 \mu\text{M}$) and uncompetitive ($K_i = 500 \mu\text{M}$) inhibition kinetics respectively. This appears to be the first phenol sulphotransferase to accept tyrosine as substrate. This membrane-bound enzyme may be involved in tyrosine transport as well as detoxification.

INTRODUCTION

When *Euglena* mitochondria are incubated with $^{35}\text{SO}_4^{2-}$ and a source of ATP, tyrosine *O*⁴-[^{35}S]sulphate [$\text{Tyr}(^{35}\text{SO}_3\text{H})$] is found in the surrounding medium (Saidha et al., 1989). Incubation of *Euglena* mitochondria or chloroplasts with adenosine 3'-phosphate 5'-phospho[^{35}S]sulphate (PAP³⁵S) also results in the accumulation of $\text{Tyr}(^{35}\text{SO}_3\text{H})$ in the medium surrounding the organelle. Since the tyrosine is supplied from endogenous sources and the activity is membrane-bound, the secretion of $\text{Tyr}(\text{SO}_3\text{H})$ by the organelles could represent the activity of a system active in tyrosine transport and/or detoxification.

Although $\text{Tyr}(\text{SO}_3\text{H})$ is known as an excretory product in animals (De Meio, 1975; Roy, 1981), the enzyme system forming this compound has been elusive. PAPS:phenol sulphotransferases or arylsulphotransferases (EC 2.8.2.1) active in detoxification (see Jakoby et al., 1980; Roy, 1981; Ramaswamy and Jakoby, 1987) generally have rather broad specificities for the phenols they will use as sulphate acceptors. Although in some cases tyrosine methyl ester is used as a substrate (Banerjee and Roy, 1968; Barford and Jones, 1971), activity is not found with free tyrosine (Jakoby et al., 1980). For this reason the nature of the system in *Euglena* organelles forming $\text{Tyr}(\text{SO}_3\text{H})$ from tyrosine and PAPS is of interest and led us to purify the enzyme and determine its properties. As we will show, there appears to be a single PAPS:phenol sulphotransferase in aplastidic *Euglena*, and the best substrate for this enzyme is tyrosine, although certain other phenols are active as well. For this reason the enzyme is referred to as phenol (tyrosine) sulphotransferase in the text. Other properties of the highly purified enzyme are presented and discussed. A brief abstract of part of this work has appeared (Saidha and Schiff, 1991).

MATERIALS AND METHODS

Cell culture and isolation

W_{10}BSmL , an aplastidic (Osafune and Schiff, 1983) mutant of *Euglena gracilis* Klebs var. *bacillaris* Cori, was inoculated from dark-grown slants or a stock culture under green safe lights (Schiff, 1972), and grown in darkness at 26 °C. The cultures were shaken (110 cycles/min) in 2-litre Erlenmeyer flasks containing 1 litre of Hutner's pH 3.5 medium (Greenblatt and Schiff, 1959) with low vitamin B₁₂ and the trace-element composition described previously (Saidha et al., 1989). Cells in the late exponential phase (5 days after inoculation) were isolated by centrifugation at 370 g for 5 min, washed with distilled water and stored at -20 °C. The cell pellets (435 g wet wt.) obtained from 87 litres of culture were thawed, combined and resuspended in 400 ml of Buffer I (containing 10 mM Tris/HCl, pH 7.4, and 0.25 M sucrose). This suspension was then divided into four equal portions which were frozen at -20 °C.

Enzyme purification procedure

A summary of the steps involved in the purification scheme is presented in Table 1. Unless otherwise specified all steps were conducted at 4 °C.

1. Preparation of crude protein extract from whole cells

The method for the preparation of crude protein extract from whole cells has been described (Li et al., 1991) and was adapted with some modifications. Each of the four aliquots of cell suspension in Buffer I frozen at -20 °C was thawed below 10 °C. Each was subjected to three cycles of freezing (in liquid N₂) and thawing (in cold water) and was then centrifuged at 10000 g for

Abbreviations used: PAPS, adenosine 3'-phosphate 5'-phosphosulphate; PAP, adenosine 3',5'-bisphosphate; $\text{Tyr}(\text{SO}_3\text{H})$, tyrosine *O*⁴-sulphate.

* To whom correspondence should be addressed.

Table 1 Purification of *Euglena* phenol sulphotransferase

Step	Total activity (nmol/min)*	Protein (mg)	Specific activity (nmol/min per mg of protein)	Purification (fold)	Recovery (%)
Freeze-thaw cell homogenate	4789	145700	0.03	0	100
Freeze-thaw supernatant fraction	2915	9805	0.29	9	61
(NH ₄) ₂ SO ₄ (30–60% satn.)	2710	860	3.16	98	56
Affi-Gel Blue	2546	222	11.43	357	53
DEAE-cellulose	2473	131	18.87	590	51
Hydroxyapatite	562	14	40.18	1255	12
First Bio-Gel	68	0.48	140.17	4394	1.4
Second Bio-Gel	60	0.29	205.33	6416	1.25

* L-Tyrosine and PAPS as substrates (see the Materials and methods section).

10 min. The supernatant fractions thus obtained were decanted and combined and comprise supernatant 1. Each of the pellets remaining from the centrifugation was resuspended in 100 ml of fresh buffer I and subjected to three cycles of freezing and thawing, followed by centrifugation; the combined supernatant fractions comprise supernatant 2. The entire procedure was repeated a third time to obtain supernatant 3. The three supernatant fractions were combined and centrifuged at 100000 *g* overnight. The supernatant fraction thus obtained is the crude protein extract and was used for purification of phenol sulphotransferase.

Cell suspensions subjected to three cycles of freezing and thawing but without subsequent centrifugation were assayed to obtain an estimate of the initial cellular enzyme activity.

2. (NH₄)₂SO₄ fractionation

Solid (NH₄)₂SO₄, 17.6 g/100 ml, was added to the crude protein extract with stirring over the course of 30 min to reach 30% saturation. After an additional 30 min, the suspension was centrifuged at 10000 *g* for 1 h and the pellet was discarded. Solid (NH₄)₂SO₄ (19.8 g/100 ml) was then added to reach 60% saturation. The suspension was re-centrifuged. This pellet contained most of the phenol sulphotransferase activity and was dissolved in Buffer I. The solution was dialysed overnight against two 4-litre changes of the same buffer.

3. Affi-Gel Blue chromatography

The dialysed protein was applied, at a flow rate of 100 ml/h, to a column (2 cm × 33 cm) of Affi-Gel Blue (50–100 mesh; Bio-Rad), previously equilibrated with Buffer I. The column was washed with 500 ml of Buffer I, followed by a linear gradient between 150 ml of Buffer I and 150 ml of Buffer I containing 0.4 M NaCl. The active protein was eluted from the column with another linear gradient composed of 250 ml of Buffer I containing 0.4 M NaCl and 250 ml of Buffer I containing 2 M NaCl. Fractions (8.1 ml each) were collected and assayed for protein and enzyme activity.

A large amount of the protein loaded on to the column was removed in the initial column washings, but no enzyme activity was observed in these fractions. The enzyme activity appeared as a broad peak eluted in the 0.4–2 M salt gradient. The active fractions were pooled and dialysed as described above against two 4-litre changes of Buffer I.

4. DEAE-cellulose

The pH of the dialysed protein solution from the previous step was adjusted to 7.9 (at 4 °C) with 1 M Tris base. This solution was then passed at a flow rate of 60 ml/h through a column (2.0 cm × 27 cm) of DEAE-cellulose (anion-exchanger; Sigma) previously equilibrated with Buffer I. The column was washed with 500 ml of Buffer I and eluted with a linear gradient between 250 ml of Buffer I and an equal volume of the same buffer containing 0.3 M NaCl; fractions (5.2 ml each) were collected. The active fractions were pooled.

5. Hydroxyapatite

The pooled and active protein solution obtained after purification on DEAE-cellulose was dialysed against two 4-litre changes of Buffer II [containing 10 mM potassium phosphate, pH 6.8, 250 mM sucrose and 0.02% (w/v) Na₂S₂O₃]. The dialysed solution was applied to a column (2.5 cm × 10 cm) of hydroxyapatite (Bio-Gel HTP; Bio-Rad) that had been previously equilibrated with Buffer II at a flow rate of 25 ml/h. The column was washed with 100 ml of the same buffer and eluted with a linear gradient formed between 150 ml of Buffer II and 150 ml of Buffer II containing 0.3 M potassium phosphate. Fractions (4.3 ml each) were collected and the active fractions were pooled. The pooled solution was concentrated to about 4 ml with an Amicon ultrafiltration cell using a PM-10 membrane (Millipore).

6. Bio-Gel (P-60) filtration

Half of the concentrated enzyme preparation obtained from the previous step was applied to a Bio-Gel P-60 column (1.3 cm × 75 cm) prepared in Buffer II with Na₂S₂O₃ raised to 0.05%. The same buffer was then passed through the column at a flow rate of 5 ml/h; fractions (1.6 ml each) were collected. SDS/PAGE of the combined fractions (designated as the first Bio-Gel step in Table 1) indicated the presence of a few contaminants of 35–50 kDa. These could be removed leaving the 26 kDa band (representing the enzyme; see below) by re-chromatography of the combined active fractions on the same Bio-Gel column (the combined active fractions so obtained are designated as the second Bio-Gel step in Table 1). The other half of the hydroxyapatite active eluate was treated similarly. The purified enzyme preparation at this stage was stored at 4 °C. Storage at –20 °C resulted in loss of enzyme activity.

For molecular-mass determination of the enzyme on a Bio-Gel P-60 column, the following molecular-mass markers were used: chick albumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa).

PAP³⁵S/paper-electrophoresis assay system

The phenol sulphotransferase activity was monitored throughout the course of enzyme purification by a modification of the PAP³⁵S assay system reported previously (Saidha et al., 1989). Tyrosine was routinely used as the sulphate acceptor phenol.

The method consists of incubating the enzyme (0.075–10 µg) in a 50 µl reaction mixture, containing 70 mM potassium phosphate, pH 7.0, 250 mM sucrose, 30 µM PAPS, 1×10^5 c.p.m. of PAP³⁵S, and 60 µM L-tyrosine (or other phenols at up to 4 mM) as sulphate acceptor. The reaction was started by the addition of enzyme, and after 10 min at 37 °C was terminated by chilling on ice. Any pelletable material, if present, was removed by microfugation (10000 g) for 10 min. An aliquot of the clear supernatant fluid was spotted [with or without non-radioactive Tyr(SO₃H)] on Whatman 3 MM filter paper (20 cm × 57 cm) and electrophoresis was performed in 0.02 M citrate buffer, pH 5.8, for 50 min at 1800 V. The paper was dried and exposed to X-ray film. When tyrosine was used as substrate, the radioactive spot with the same mobility as added Tyr(SO₃H) (detected by quenching at 253 nm) was marked, cut out and transferred into a scintillation vial, and radioactivity was determined in a scintillation counter (Saidha et al., 1985, 1988). When other phenols were used as substrates, the radioactive phenol sulphate formed was detected by exposure to X-ray film, and the area on paper corresponding to the radioactive spot on film was marked, cut out and counted for radioactivity as described above. Only one radioactive spot (other than those corresponding to PAP³⁵S and ³⁵SO₄²⁻) was found after incubation with each active substrate; these spots had mobilities that were not very different from that of Tyr(³⁵SO₃H). No radioactive spots other than PAP³⁵S and ³⁵SO₄²⁻ were found with inactive substrates. However, when 2-naphthol was used as substrate, a spot containing a small amount of radioactivity was found close to synthetic 2-naphthyl sulphate, but further away from 1-naphthyl sulphate; the radioactivity, however, did not coincide with either compound. This product probably arises from an impurity in the 2-naphthol, possibly another naphthol derivative, which is active in the system. 2-Naphthol itself appears to be inactive as a substrate in the present system.

Preparation of Tyr(SO₃H) and PAP³⁵S

Tyr(SO₃H) was prepared by the method of Fitzgerald et al. (1979), with modifications described previously (Saidha et al., 1989) or was purchased from Adams Chemical Co., Roundlake, IL, U.S.A. PAP³⁵S was prepared as previously described without added non-radioactive sulphate (Saidha et al., 1985).

SDS/PAGE and native PAGE

SDS/PAGE

Fully denaturing SDS/PAGE was performed according to previously described methods (Laemmli, 1970; Spano and Schiff, 1987) with some modifications. Slab gels (7 cm × 8 cm × 0.75 mm) were cast at room temperature with 3.1 ml of resolving gel monomer solution containing 9% (w/v) acrylamide. After allowing 1 h for polymerization, the prepared 1.1 ml stacking-gel monomer solution containing 4.0% (w/v) acrylamide was

layered on top of the resolving gel and allowed to polymerize. Samples were solubilized in Laemmli's solubilization buffer [containing 125 mM Tris/HCl, pH 6.8, 2.0% (w/v) SDS, 10% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue] by heating for 3 min in a boiling-water bath before application to the gel. The upper and lower buffer reservoirs contained 192 mM glycine/25 mM Tris/0.1% (w/v) SDS, pH 8.3. Electrophoresis was performed at 190 V for 45 min. Molecular-mass standards included BSA (66 kDa), egg albumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Native PAGE

Native PAGE was performed by the method of Roscoe and Ellis (1982) with modifications. A gel similar to that described for SDS/PAGE, with 7.5% and 3.0% (w/v) of acrylamide in resolving and stacking portions of the gel respectively, was used. Sucrose (60%) was included in the gel composition to preserve the enzyme activity, but β-mercaptoethanol and SDS were excluded because of their inactivating effect on the enzyme. The sample in Buffer II was applied to the gel and electrophoresis was performed at 4 °C in 25 mM Tris/192 mM glycine, pH 8.3, at 190 V for 45 min. Enzyme-activity measurements were performed on an unstained lane cut into 0.5 cm gel segments. Each segment was macerated in 50 µl of 50 mM Tris/HCl, pH 6.8, containing 250 mM sucrose. The macerate was Microfuged at 10000 g for 10 min. An aliquot of the clear supernatant fluid was used for assaying the enzyme activity by the PAP³⁵S/paper-electrophoresis assay described above, but without the addition of non-radioactive PAPS. A parallel lane was stained for comparison.

The SDS or native PAGE gels were stained for 30 min in a solution containing 40% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R. Destaining was accomplished by gently shaking the gels in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

Proteins were determined by the method of Bradford (1976), with BSA as the standard.

Experiments were done at least twice with comparable results; representative experiments are shown.

RESULTS AND DISCUSSION

Enzyme purification

Table 1 summarizes the purification of the tyrosine-utilizing phenol sulphotransferase activity from dark-grown cells of aplastidic *Euglena* mutant W₁₀. Freezing and thawing was known to be effective in releasing the enzyme from membranes in active form (Saidha et al., 1989; Saidha and Schiff, 1991) and was employed as the first step. The activity in the crude unseparated freeze-thawed cell suspension was taken as 100%. This suspension was centrifuged and the supernatant fraction, containing 61% of the activity and showing some purification, was used for subsequent steps. The protein precipitating between 30 and 60% satn. of (NH₄)₂SO₄ had 56% of the total initial activity with an increase in purity. Chromatography of the dialysed (NH₄)₂SO₄ fraction successively on Affi-Gel Blue, DEAE-cellulose and hydroxyapatite gave considerable purification (Table 1). Although we had used Sephadex chromatography as a purification step in our preliminary work (Saidha and Schiff, 1991), we have since found that exposure of the enzyme to dextran results in instability, particularly in the later stages of purification. For this reason we turned to polyacrylamide-based Bio-Gel columns,

Table 2 Substrate specificity of *Euglena* phenol sulphotransferase*

Kinetic constants were determined using the PAP³⁵S assay at pH 7.0. A PAPS concentration of 30 μ M was used for K_m and V_{max} determinations of sulphate acceptors.

Substrate	K_m (μ M)	V_{max} (nmol/min per mg of protein)
L-Tyrosine	33	55
L-Tyrosinamide	200	42
L-Tyrosine methyl ester	150	27
L-Tyrosine ethyl ester	100	45
L-Tyrosine allyl ester	370	63
L-Tyrosine <i>t</i> -butyl ester	80	42
L-Tyrosine benzyl ester	133	66
L- <i>p</i> -Hydroxyphenylglycine	714	72
Glycyltyrosine	2500	160
Tyrosylglycine	833	114
Alanyltyrosine	350	40
<i>m</i> -Nitrophenol	416	20
<i>p</i> -Nitrophenol	1250	10
<i>o</i> -Chlorophenol	500	14
<i>m</i> -Chlorophenol	400	13
<i>p</i> -Chlorophenol	550	20

* Purified through the second Bio-Gel step (see Table 1).

which maintain the stability of the enzyme. Two passes through Bio-Gel P-60 columns as the final steps resulted in a purification of over 6000-fold (Table 1).

The purified enzyme showed instability to freezing, but could be stored for at least 5 months in Buffer II at 4 °C with no more than 10% loss of activity. A similar instability of the enzyme to freezing was observed for other phenol sulphotransferases (Sekura and Jakoby, 1979). The addition of thiols at any stage in the enzyme purification either decreased the enzyme activity or had no effect.

The ratios of enzyme activities with saturating L-tyrosine, *m*-chlorophenol or *m*-nitrophenol remained fairly constant throughout enzyme purification at values that were similar to the ratios of the V_{max} values of the highly purified enzyme for these substrates that can be computed from the data in Table 2. Thus the ratios of specific activities obtained at the (NH₄)₂SO₄, Affi-Gel Blue, DEAE-cellulose, hydroxyapatite and first-Bio-Gel stages of enzyme purification (steps shown in Table 1) were 3.16, 3.57, 2.81, 2.53 and 2.65 respectively for specific activity with L-tyrosine as substrate/specific activity with *m*-nitrophenol as substrate. Similarly specific activity with L-tyrosine/specific activity with *m*-chlorophenol at each stage of purification was 3.54, 4.21, 3.26, 3.13 and 2.11 respectively and for *m*-nitrophenol/*m*-chlorophenol was 1.12, 1.18, 1.16, 1.24 and 0.80. The corresponding ratios computed from the data in Table 2 for the ratio of V_{max} of the purified enzyme with various substrates are 2.75 for L-tyrosine/*m*-nitrophenol, 4.23 for L-tyrosine/*m*-chlorophenol and 1.54 for *m*-nitrophenol/*m*-chlorophenol. Considering the differing purity of the various fractions and possibilities for uncontrolled influences on the activities from one enzyme fraction to the next, the ratios are remarkably similar, supporting the view that the same enzyme is responsible for all three activities throughout purification. Using L-tyrosine as the assay substrate, the enzyme was eluted as a single symmetrical peak from all columns used; there was no indication of the presence of more than one enzyme activity. Taken together, the data indicate that there is only one phenol sulphotransferase in cells of *Euglena*

Figure 1 Purified phenol (tyrosine) sulphotransferase from *Euglena* on native PAGE stained with Coomassie Blue and the corresponding relative activity profile

Enzyme, purified through the second Bio-Gel step (see Table 1) (1.5 μ g of protein/lane) was subjected to electrophoresis and the activity measurements performed in gel slices as indicated in the Materials and methods section. The activity of the highest peak represents 1230 c.p.m./10 min. The upper 'band' seen in the gel lane is the result of differential staining and destaining of the stacking and resolving gels and indicates the point at which the two gels meet.

mutant W₁₀BSmL using L-tyrosine and the other substrates indicated in Table 2 as sulphate acceptors.

A single band was detected when the purified enzyme was subjected to native PAGE (Figure 1) and it was coincident with the phenol (tyrosine) sulphotransferase activity. When this active band was eluted from the native gel and subjected to electrophoresis on an SDS-containing gel, a single band at a molecular mass of 26 kDa was obtained (Figure 2). In agreement, a molecular mass of 26 kDa was obtained from Bio-Gel P-60 chromatography of the purified enzyme. Electrophoresis of the purified enzyme directly on SDS-containing gels (Figure 3) also yielded the same molecular mass. The purified phenol (tyrosine) sulphotransferase from *Euglena* is a monomer with a slightly lower molecular mass than the subunits of the usual phenol sulphotransferases that do not utilize L-tyrosine as substrate. These soluble enzymes commonly have molecular masses in the range of 60000–70000 and when purified consist of two identical subunits (Sekura and Jakoby, 1979).

The phenol (tyrosine) sulphotransferase activity is found in the membranes of purified mitochondria and chloroplasts from *Euglena* (Saidha et al., 1989), and since an aplastidic mutant was used as the enzyme source in the present work, the purified enzyme probably represents the mitochondrial activity. However, the possibility that the enzyme is also present at other cellular sites is not ruled out.

Enzyme kinetics and inhibition

The purified phenol (tyrosine) sulphotransferase from *Euglena* shows normal Michaelis–Menten kinetics with L-tyrosine and PAPS (Figures 4a and 4b). The apparent K_m for L-tyrosine is 33 μ M (PAPS concn. 30 μ M) and for PAPS is 15 μ M (L-tyrosine

Figure 2 Electrophoresis of the *Euglena* phenol (tyrosine) sulphotransferase band, excised from a native PAGE gel, on SDS/PAGE

Lanes 1 and 2 show the Coomassie stained bands from two separate lanes of the native-PAGE gel (prepared as in Figure 1), which were excised, macerated and subjected to electrophoresis on a SDS/9%-PAGE gel. Lane 3 shows the separation of standard molecular-mass markers as indicated in the Materials and methods section.

Figure 3 SDS/PAGE pattern of purified phenol (tyrosine) sulphotransferase from *Euglena*

Lane 1, standard molecular-mass markers as indicated in the Materials and methods section. Lane 2, purified protein (1.5 µg) from the second Bio-Gel step (see Table 1).

concn. 60 µM). Adenosine 3',5'-bisphosphate (PAP), a product, is a competitive inhibitor (K_i 20 µM) of the phenol sulphotransferase reaction (Figure 4c), using PAPS as the variable substrate and L-tyrosine at 60 µM. The other product, Tyr(³⁵SO₃H), is an uncompetitive inhibitor (K_i 500 µM) of the reaction with L-tyrosine, with L-tyrosine as the variable substrate and PAPS at 30 µM (Figure 4d).

PAPS at 30 µM was used throughout. This concentration was well within the range where rate did not increase with substrate concentration, but avoided any inhibition by PAPS, which began at twice this concentration.

Like other phenol sulphotransferases (Sekura and Jakoby,

Figure 4 Initial-velocity kinetics of the phenol (tyrosine) sulphotransferase reaction in the absence or presence of fixed concentrations of inhibitors

Euglena enzyme from the second Bio-Gel step (see Table 1) was used. Activities were measured by the PAP³⁵S assay described in the Materials and methods. (a) $1/v$ plotted against $1/[\text{tyrosine}]$; (b) $1/v$ against $1/[\text{PAPS}]$; (c) $1/v$ against $1/[\text{PAPS}]$ at various fixed concentrations of PAP (●, 100; ○, 40; □, 20 µM); (d) $1/v$ against $1/[\text{tyrosine}]$ at various fixed concentrations of Tyr(SO₃H) (●, 3; ○, 2; □, 1 mM).

Figure 5 Influence of pH on the activity of *Euglena* phenol (tyrosine) sulphotransferase purified through the second Bio-Gel step (see Table 1)

Phosphate (□) was used at 70 mM, Tris/HCl (◆) at 50 mM and glycine (■) at 50 mM.

1979; Roy, 1981) the phenol (tyrosine) sulphotransferase shows a complex pH curve with a major peak in the vicinity of pH 7.0 (Figure 5). Phosphate appears to be better than Tris/HCl for achieving maximum activity in this system.

Substrate specificity

Because, to our knowledge, this appears to be the first phenol sulphotransferase described that uses free L-tyrosine as substrate, further experiments were done to obtain some idea of the range of phenols that are utilized as sulphate acceptors. The kinetic

parameters (apparent K_m and V_{max}) of compounds found to be active as substrates are shown in Table 2. Substrates that were found to be inactive are simply mentioned in the text.

The substrate with the lowest K_m was L-tyrosine (Table 2). Replacement of the alanine residue by glycine (in L-*p*-hydroxyphenylglycine) raised the K_m , but the compound was still active. However, D-tyrosine and D-*p*-hydroxyphenylglycine were inactive, indicating that the enzyme accepts only tyrosine derivatives with the L configuration. The activity with L-tyrosine was not inhibited by the addition of equimolar D-tyrosine (results not shown).

Extra substituents on the ring [as in 3,5-dinitro-L-tyrosine or L-dopa (L-3,4-dihydroxyphenylalanine) or the hydroxy group in the wrong position on the ring (as in *o*-DL-tyrosine and *m*-DL-tyrosine)] resulted in a lack of activity as substrate. It is assumed that, like L- and D-tyrosine, the D form does not inhibit the activity, if present, of the L form.

Substitution of the free carboxy group of tyrosine by formation of an amide or ester linkage yields an active substrate (Table 2), even though some of the ester groups are fairly bulky. In all cases the K_m is higher than with L-tyrosine. However, loss of the carboxy carbonyl group as in L-tyrosinol (L-2-amino-3-*p*-hydroxyphenylpropanol) or tyramine yields an inactive compound. 3-Hydroxytyramine is also inactive, either for this reason or because of the extra substituent on the ring (see above).

Glycyltyrosine and alanyltyrosine, which are linked by peptide bonds through the amino group of tyrosine, are active substrates with rather high K_m values compared with free tyrosine, but eventually yield a substantial V_{max} . Tyrosylglycine, linked through the carboxy group of tyrosine, is also active (Table 2), but tyrosylalanine is not. Perhaps the longer alanine group prevents an effective interaction with the enzyme.

Derivatives of tyrosine lacking the amino group such as DL-*p*-hydroxyphenyl-lactic acid, 3-(*p*-hydroxyphenyl)propionic acid and *p*-hydroxyphenylpyruvic acid are all inactive.

A number of phenols not closely related to tyrosine were also tried. *o*-, *m*- and *p*-Chlorophenols were all active, with K_m values higher than that for tyrosine (Table 2). *m*- and *p*-Nitrophenols were also active, with a much higher K_m for the *p* compound, but the *o* compound was inactive. The other phenols tested were all inactive including phenol, *o*-, *m*- and *p*-cresols, 1-naphthol, 2-naphthol, D-thyroxine, L-thyroxine and *N*-acetyl-5-hydroxytryptamine ('serotonin'). As noted in the Materials and methods section, the low activity previously seen with 2-naphthol (Saidha and Schiff, 1991) can be attributed to the sulphonation of an impurity in the 2-naphthol preparation. It is interesting that all of the cresols (methylphenols) are inactive, including *p*-cresol, which can be thought of as a tyrosine analogue lacking the terminal two carbons and functional groups of the alanine moiety.

L-Tyrosine and related compounds are very good substrates for the *Euglena* phenol (tyrosine) sulphotransferase; free L-tyrosine gave the lowest K_m of all phenol acceptors tested (Table 2). However, the *Euglena* enzyme will use other unrelated phenols (such as the *o*-, *m*- and *p*-chlorophenols and *m*- and *p*-nitrophenols) as substrates with higher K_m values. It is interesting that an enzyme with such exacting requirements for configuration and structure of the tyrosine molecule accepts compounds such as the chlorophenols and nitrophenols as substrates which are apparently unrelated. However, the K_m values for these unrelated phenols are not very different from those obtained with other

phenol sulphotransferases which do not accept free tyrosine as substrate (Sekura and Jakoby, 1979).

The ability to use L-tyrosine as substrate may have necessitated a restriction in the range of other substrates, however, since the *Euglena* enzyme will not use a variety of substrates readily accepted by other phenol sulphotransferases such as naphthols, cresols and a number of other compounds such as *N*-acetyl-5-hydroxytryptamine. In general, the usual phenol sulphotransferases from mammalian sources appear to accept a much broader spectrum of substrates than the *Euglena* enzyme. It is of interest in this connection that the mammalian enzymes that use L-tyrosine methyl ester have rather high K_m values for this substrate (2900–5000 μ M) (Banerjee and Roy, 1968; Barford and Jones, 1971) compared with the *Euglena* enzyme (Table 2). The usual mammalian phenol sulphotransferases appear to have evolved as enzymes of detoxification, and evolutionary selection would favour a wide range of substrate acceptance. The utilization of free L-tyrosine (and similar compounds) as substrates may have been related to both detoxification of excess L-tyrosine and, perhaps, to an involvement of the *Euglena* enzyme in tyrosine transport (Saidha and Schiff, 1991).

Whatever the reason, the evolution of a phenol sulphotransferase that utilizes L-tyrosine and related compounds appears to have necessitated a restriction in the range of other phenol acceptors that can be utilized and may be one reason why the usual phenol sulphotransferases do not use L-tyrosine as substrate. The *Euglena* enzyme appears to be the first phenol sulphotransferase that has been found to act on free L-tyrosine. It would be interesting to know whether this type of phenol sulphotransferase is found in other organisms, including those which have high titres of the more usual detoxifying enzymes.

This work was supported by grants from the National Science Foundation. J. A. S. is Abraham and Etta Goodman Professor of Biology. The technical assistance of Miss Nancy O'Donoghue is appreciated. We also thank Gabriel Gonzales and Brad Andelman for their contributions to this work.

REFERENCES

- Banerjee, R. K. and Roy, A. B. (1968) *Biochem. J.* **151**, 573–586
- Barford, D. J. and Jones, J. G. (1971) *Biochem. J.* **123**, 427–434
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- De Meio, R. H. (1975) in *Metabolism of Sulfur Compounds* (Greenberg, D. M., ed.), pp. 287–358, Academic Press, New York and London
- Fitzgerald, J. W., Maca, H. W. and Rose, F. A. (1979) *J. Gen. Microbiol.* **111**, 407–415
- Greenblatt, C. L. and Schiff, J. A. (1959) *J. Protozool.* **6**, 23–28
- Jakoby, W. B., Sekura, R. D., Lyon, E. S., Marcus, C. J. and Wang, J.-L. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., ed.), vol. 2, pp. 199–229, Academic Press, New York
- Laemmli, U. K. (1970) *Nature (London)* **307**, 478–480
- Li, J., Saidha, T. and Schiff, J. A. (1991) *Biochim. Biophys. Acta* **1078**, 68–76
- Osafune, T. and Schiff, J. A. (1983) *Exp. Cell Res.* **148**, 530–535
- Ramaswamy, S. G. and Jakoby, W. B. (1987) *Methods Enzymol.* **143**, 201–207
- Roscoe, T. J. and Ellis, R. J. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., Hallick, R. B. and Chua, N.-H., eds.), pp. 1015–1028, Elsevier Biomedical Press, Amsterdam, New York and Oxford
- Roy, A. B. (1981) in *Sulfation of Drugs and Related Compounds* (Mulder, G. J., ed.), pp. 83–130, CRC Press, Boca Raton, FL
- Saidha, T. and Schiff, J. A. (1991) *Plant Physiol.* **96S**, 155
- Saidha, T., Stern, A. I., Li, J. and Schiff, J. A. (1985) *Biochem. J.* **232**, 357–365
- Saidha, T., Na, S.-Q., Li, J. and Schiff, J. A. (1988) *Biochem. J.* **253**, 533–539
- Saidha, T., Hanfstring, U. and Schiff, J. A. (1989) *Arch. Biochem. Biophys.* **272**, 237–244
- Schiff, J. A. (1972) *Methods Enzymol.* **24**, 321–322
- Sekura, R. D. and Jakoby, W. B. (1979) *J. Biol. Chem.* **254**, 5658–5663
- Spano, A. J. and Schiff, J. A. (1987) *Biochim. Biophys. Acta* **894**, 484–498